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Antagonistic interaction of Pseudomonas aeruginosa and Candida albicans.

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ANTAGONISTIC INTERACTION OF PSEUDOMONAS AERUGINOSA AND CANDIDA ALBICANS

A Dissertation Presented

By

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DEDICATION

To my wife Barbara, and my sons Dan and Steve--
This accomplishment is as much theirs as mine.

ANTAGONISTIC INTERACTION OF *PSEUDOMONAS AERUGINOSA* AND *CANDIDA ALBICANS*

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Pseudomonas aeruginosa of human origin has been found to be antagonistic to *Candida albicans* from the same habitat. *In-vitro* studies showed the presence of an anti-biotic substance produced by *Pseudomonas*. Clinical isolates of these organisms were tested in associative cultures and while the *Candida* isolates were uniformly inhibited, *Pseudomonas spp.* displayed variability in their inhibitory capacity. Sterile filtrates on spent *Pseudomonas* broth cultures, when spotted on the surface of *Candida* seeded overlays, induced discrete zones of inhibition. Two compounds were responsible: pyocyanine, which exhibits a wide zone of incomplete inhibition, and phenazine-1-carboxylic acid, which exhibits a narrow zone of complete inhibition. It appears that each compound reinforces the other and together these are responsible for the inhibitory effect observed with the crude filtrate.

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I N T R O D U C T I O N

The advances made in medicine during the past decade have been accompanied by an increasing incidence of opportunistic infections. Patients acquiring infections are considered immunologically incompetent and are unable to defend themselves against the most innocuous organisms. The indigenous microflora of the healthy individual becomes life-threatening to those who undergo extensive diagnostic and therapeutic regimens and prolonged hospitalization. Recovery from the underlying disease is aggravated by the complication of infection for which medication is often ineffective. Antibiotic resistance among opportunistic organisms is widely recognized and while a few specific anti-microbial agents are available, their toxicity restricts general usage.

Two particularly evident opportunists are Candida albicans, a yeast, and Pseudomonas aeruginosa, a bacterium. Presently, there is an increasing incidence of infections due to these organisms among the severely debilitated receiving broad spectrum antibiotic therapy. Eradication of the normal flora colonizing the respiratory and intestinal tracts provides an ideal non-competitive habitat for these opportunists to proliferate.

The yeast ordinarily occurs in small numbers in the upper respiratory tract, an environment heavily laden with a competitive flora. When this habitat is altered, C. albicans multiplies and may progress to the lungs. Such systemic infections are difficult to control. Of the two candida specific drugs available, nystatin and amophotericin B, only the latter is effective for systemic infection. This agent, however, is exceedingly toxic and is not generally administered.

The bacterium, Pseudomonas, is somewhat similar; it is commonly found in moist areas of the inanimate environment. From these sources colonization of the human respiratory and intestinal tract readily occurs. Its significance when found within the healthy host is problematic, although traditionally, it has been considered part of the normal flora. These and other opportunists present a diagnostic and therapeutic dilemma to the physician. Diagnosis must consider not only the organism, their numbers and relative proportion, but more importantly the ability of the patient to resist their progress.

Since both Candida albicans and Pseudomonas aeruginosa are (a) ubiquitous, (b) opportunists, (c) resistant to antibiotics and (d) have the same temperature preference, they should, in theory, be regularly isolated from the immunologically susceptible patient. We have

observed that this is not the case. Either one or the other, but rarely both, are isolated from the same individual. This observation led to studies of their associative behavior, which discloses that Pseudomonas is antagonistic towards Candida, and attempts to determine the agents responsible for this antagonism.

L I T E R A T U R E R E V I E W

It is exceedingly unlikely that two populations growing in the same habitat do not interact. The prudent observation made by Lucas (23) in 1949 that, "Mere toleration is biologically and statistically improbable," is becoming increasingly evident. Neutralism is the term used to describe associations in which different biological species coexist, each indifferent to the others presence. Such association probably occurs only when the populations are physically far apart or the physiological requirements are so different that neither organism exerts an influence on the other. As a result, neutralistic associations have received little interest and have not been systematically studied (4).

The study of microbial associations has revealed a variety of possible interactions. Unfortunately, there is a tendency to consider these interactions of neutralism, commensalism, antagonism and synergism as independent and unchanging. This is a major fault, especially in the assessment of the human microflora, in that rigid criteria dealing only with benefit or harm are oversimplifications of complex interactions. This may be illustrated by the commensals which colonize the respiratory tract, especially

the streptococci and yeasts. While these two organisms are everpresent they very rarely cause disease. When disease does occur it's primarily due to changes on the part of the host and not to the inherent virulence of the organism. In light of this, it is becoming clear that the terms "pathogen" and "non-pathogen" may also be an oversimplification and should be replaced with terms which consider both the host and the organism (9). Present understanding of microbial interactions within the human body is limited, since it is an ecological problem that must consider not only the indigenous flora but exogenously acquired organisms as well. Unfortunately, most of the present knowledge regarding microbial interactions is the result of in-vitro and bovine rumen investigations and is extremely limited.

A particularly attractive concept of interaction has been that of antagonism; associations characterized by suppression of one organism for the benefit of the other. In the past decade there has been renewed interest in the prospect of promoting microbial antagonism not only as a means of suppressing infectious diseases of man, but also bacterial control of insects, vermin and rodents. Due to the indiscriminate use of pesticides and the problems attributed to these chemicals, biological control systems are rapidly gaining favor and much effort is being expended at the present time developing appropriate and efficient

biological control systems.

A considerable amount of work has been reported utilizing both in-vivo and in-vitro conditions in the control of infectious diseases by competitive inhibition. The in-vivo studies have demonstrated unequivocally that bacterial interference can be used to augment man's ability to resist disease.

The terminology used in these studies generally includes the following: (1) Test strain--the organism being antagonized, suppressed or killed; (2) Effector strain--the organism responsible for the antagonism and (3) Bacterial interference--the phenomenon of antagonism by one organism toward another and synonymous with competitive interaction.

The concept of infectious disease control by promoting competitive interaction was first introduced in 1885 by Contani (8) who attempted treatment of tuberculosis by replacement of the tubercle bacillus with a mixture of indigenous species. In 1906 Emmerich (8) injected beta-hemolytic streptococci with the prospect of preventing the spread of anthrax. Florey's review (8) of the use of micro-organisms for therapeutic purposes examines the research of Metchnikoff and others at the beginning of this century and discusses the possible reasons for the failure of the concept. On the other hand, success of this concept is most dramatically illustrated by the experience of Wagner and Jauregg

who employed malarial fever therapy for the treatment of syphillis (6, 32, 43).

This early era of natural therapy ended in the 1930's with the development of sulfa drugs and penicillin. At this time, interest in microbial antagonism as a means of therapy was pre-empted by the antibiotic era that followed. The concept received little attention until the 1960's when two discouraging shifts were observed by the clinical microbiologist: (1) antibiotic resistant strains of bacteria were becoming more prevalent and (2) gram negative bacilli, formerly considered innocuous, were more frequently documented as etiologic agents of infectious disease. These two trends indicated the limitations of chemotherapy and a renewed interest in biological control followed.

Shinefield et al (34, 35) in 1963, were among the first to reinvestigate biological control when they implanted avirulent Staphylococcus aureus strain 502A on the nasal mucosa and the umbilicus of newborn infants and successfully interrupted transmission of epidemic strains among the nursery population. The same avirulent strain was also successfully established in the nasal mucosa of adults who had previously carried virulent strains of S. aureus. Staphylocci have also been shown to inhibit a wide variety of other gram positive bacteria including Corynebacterium diptheriae and Nocardia species. Mycobacterium smegmatis, Bacillus, Clostridium and other Staphylococcus species

have been evaluated in competition with S. aureus and were found susceptible in-vitro (11).

Monosporium apiospermum,a fungus, has been reported by Cormane et al (7) to be inhibited by both S. aureus and S. albus. It was similarly demonstrated by Prelle that 28 of 30 strains of Neisseria, gram negative cocci, were inhibited by all 10 strains of S. aureus used in his studey (29). Streptococci, including both the hemolytic non-hemolytic types other than enterococci, have been reported susceptible to the antagonism of Staphylococcus species (36).

Among the protozoa, the blood flukes have been reported to be susceptible to the effects of certain enteric bacilli, Klebsiella pneumoniae injected into mice has been shown to effectively control Shistosomiasis. Using mice with an established infection of Shistosoma mansoni, Ottens and Dickerson (27) demonstrated that intraperitoneal injection of K. pneumoniae destroyed the fluke in 3 to 4 days. A positive relationship between the multiplication of the bacteria in the ceacum and the rapid death of the parasite was demonstrated.

Another group of antagonistic bacteria are the streptococci, especially members of the viridans group. Sanders (31) demonstrated that Streptococcus pyogenes is is antagonized by viridans streptococci. Neither the

phase of growth nor the inoculum size affected the in-vitro antagonism. While all group A streptococci were inhibited by active viridans strains, he reported significant differences in the antagonistic capacity of S. viridans. He ascribed this to nutrient depletion and accumulation of acidic end products. In-vitro studies of children's throat flora was the basis for his hypothesis that the qualitative composition of the viridans strains inhabiting the pharynx is a major determinant of an individual's resistance to group A streptococcal infections.

Similar observations of the inhibitory capacity of the viridans streptococci were made by Sprunt (37). Evidence from 10 patients undergoing massive antibiotic therapy indicated the elimination of viridans streptococci and replacement with Hemophilus influenzae and yeasts.

Kalmanson et al (17) using an agar overlay technique to demonstrate antagonism were able to effect inhibition of microbial L-forms. Emoloying paper discs saturated with S. faecalis broth, the discs were placed on a nutrient agar base layer. An agar overlay seeded with the L-form organism was then applied over the base layer.

Among the earliest investigations of antagonistic interactions were those with Pseudomonas as effector strains. Emmerich and Low (8) using spent cultures of Pseudomonas found them effective against Bacillus anthracis, Vibrio

cholerae, Salmonella typhi, C. diptheriae, Pasteurella pestis and a variety of staphylococci. They suggested that the agent responsible was the enzyme "pyocyanase." It was later determined that several crystalline pigments belonging to the phanazine class of compounds were responsible for the antagonism observed by the earlier workers (33). Pyocyanine, the best known of these compounds, is a blue pigment, extractable with $CHCl_3$ and mildly bacteriostatic. Like the other phenazine pigments, it has never been used clinically as an antibiotic due to its toxicity.

In light of the present dilemma concerning the usefulness and limitations of antibiotics, it is perhaps the organism and not the extracted products that deserve greater consideration. Pseudomonas, in this regard, has been isolated with regularity from the respiratory tract of healthy subjects. Sutter et al (38) recovered this organism from 6.6% of the oral cavities of 350 normal subjects. The intact Pseudomonas cell with its capacity to synthesize a variety of phenazine pigments, each with considerable anti-microbial activity, might be a significant factor in natural resistance to disease.

That the usefulness of extracted antibiotics produced by pseudomonads is limited, was keenly observed by Rosebury (30) who stated that "--the search for useful antibiotics among indigenous effectors has been entirely fruitless."

This seems especially significant when one considers that Ps. aeruginosa was the first indigenous microorganism to disclose antibiotic effects (7). Nevertheless, this organism, as an effector strain, has the capability to produce antibiotics while most indigenous effectors either elaborate major metabolites, such as ethanol, or compete for vital nutrients. Also significant is the ability of Pseudomonas species to synthesize pyocines, which are active against other strains of the same species.

Candida, with its well-known capacity to inhabit man as either a pathogen or commensal, is another organism which must be included in this consideration of interactive phenomena (21). Of particular concern is its widespread resistence to antibiotics. The new drugs presently available and relatively effective against Candida are Mycostatin and Amphotericin B. Mycostatin is limited to superficial infections; cases often as effectively treated with potassium permanganate or crystal violet preparations. Amphotericin B is used for systemic candidiasis but its toxicity severely restricts its usage. For these reasons the physician is reluctunt to prescribe these drugs and tends to manage a regimen which treats the underlying disease to which Candida is often secondary (13, 27).

As a participant in microbial interactions, Candida is known to exhibit both stimulative and inhibitory effects.

The vitamin components and the amino acids elaborated are apparently responsible for the stimulatory effect (29). Lactobacillus species and Neusseria catarrhalis are known to benefit from a wide variety of associations, one of which is with C. albicans (30, 42). Escherichia coli is similarly stimulated by the presence of C. albicans. Candida, on the other hand, is inhibited by its presence. Rosebury et al (30) reported that E. coli grew luxuriantly in association with Candida on Douglas Lactate agar. When E. Coli was cultivated separately, no growth was observed.

Inhibition of Candida may be ascribed to competition for nutrients. In a study using fresh saliva, sterile and contaminated, growth of Candida was determined to be directly proportional to the concentration of salivary glucose. Peak growth of Candida was observed on sterile saliva and could only be induced on contaminated saliva if glucose was added (20). However, with the Candida-Lactobacillus association, it has been observed that Candida inhibition was due to the concentation of excess lactic acid and hydrogen ions (42).

As an antagonist, Candida may produce an antibiotic which inhibits N. gonorrhea. Genital tract specimens often contain both of these organisms and yet growth of Neisseria is often diminished or entirely prevented when in competition with Candida, a phenomenon not evident when Candida is absent.

An inhibitory factor not yet identified but presumably an antibiotic has been extracted with tertiary butanol (14).

The significance of metabolites such as ethyl alcohol as inhibitory agents when synthesized in-vivo has not been reported although blood alcohol levels of 0.02% EtOH have been found post mortem apparently synthesized by C. albicans (36).

In the studies of antagonistic interaction it is apparent that three possible mechanisms of inhibition may be operative: (1) depletion of nutrients essential to the associate organism, (2) elaboration of a major metabolite with the creation of a restrictive physiological condition and (3) synthesis of an antibiotic substance. While any one of these may not be difficult to demonstrate in-vitro, the evidence for their in-vivo action is indirect and circumstantial.

M A T E R I A L S A N D M E T H O D S

Organisms

Most of the microbial cultures employed in this study were obtained as fresh clinical isolates. The bacterial and yeast strains studied are listed in Table 1 and were maintained on slants of Stock Culture agar (Difco), at ambient air temperature and transferred monthly.

The organisms were characterized using standard bacteriological tests as described in the Manual of Clinical Microbiology by Blair et al (2). All media were freshly prepared from a dehydrated base (Difco) and reactivity confirmed before use.

The Candida isolates were initially isolated on Tryptose Blood agar (TBA, Difco) and transferred to Sabouraud Dextrose agar (Difco) for separation. After 24 hours incubation at 37C, purity was ascertained by visual inspection and a single colony used as inoculum for the following procedures: germ tube production, BIGGy agar (Difco) reaction, carbohydrate utilization, chlamydospore production and urease activity. Only those isolates producing reactions consistent with C. albicans were retained in the culture collection.

TABLE 1

CLINICAL ORIGINS OF EFFECTOR
AND TEST ORGANISMS

Effector Strain	Origin
Ps. aeruginosa 69467	respiratory tract
Ps. aeruginosa 69326	urinary tract
Ps. aeruginosa 68114	respiratory tract
Ps. aeruginosa 100	respiratory tract
Ps. aeruginosa 68084	tracheostomy incision
Ps. aeruginosa 69616	urinary tract
Ps. aeruginosa 67650	respiratory tract
Ps. aeruginosa 68394	respiratory tract
Ps. aeruginosa 69376	tracheostomy incision
Ps. aeruginosa 69322	respiratory tract
Ps. aeruginosa 69386	urinary tract
Test Strain	
C. albicans 68656	respiratory tract
C. albicans 68443	genital tract
C. albicans 67324	urinary tract
C. albicans 67578	urinary tract
C. albicans 68340	respiratory tract
C. albicans 67624	genital tract
C. albicans 67626	respiratory tract
C. albicans 68236	respiratory tract
C. albicans 68277	urinary tract
C. albicans 68366	urinary tract
C. albicans 68298	gastrointestinal tract
C. albicans 68655	respiratory tract

The Pseudomonas isolates were also recovered from TBA plates inoculated with clinical material. Colonies resembling Pseudomonas were transferred to Meuller Hinton agar (MH, Difco) and after 24 hours incubation a single colony was used as inoculum for the following determinations: reaction in O/F Medium (Difco) containing glucose, motility in a hanging drop preparation, ketogluconate exidation (Key Scientific) and cytochrome oxidase reaction using p-amino dimethylaniline oxalate reagent. Confirmed isolates of Ps. aeruginosa were then transferred to the stock culture collection for further study.

Associative Cultures

Initially the interference phenomenon was observed on solid media. Peptone agar plates were inoculated with a single diametric streak of Pseudomonas, after 24 hours incubation the agar was inverted using the "flip-flop" method reported by Kekessey and Piquet (15). The agar was loosened with a sterile spatula and inverted into the lid of the petri dish. Candida was then streaked onto the exposed agar surface perpendicular to the original Pseudomonas streak. The plate was again incubated at 37 C and the inhibition noted.

A variety of in-vitro techniques, utilizing either liquid or solid media, have been devised to demonstrate interference among microorganisms (29). In this phase,

liquid cultures were utilized in three studies in order to evaluate the routine methods employed to demonstrate this antagonism.

The first quantified the growth of Candida on sterile spent Pseudomonas broth and also the growth of Pseudomonas on similarly prepared Candida broth. A 500 ml Erlenmeyer flask containing 100 ml of Brain Heart Infusion (BHI, Difco) was inoculated with a BHI culture of Pseudomonas aeruginosa 100 that was incubated overnight at 37 C. A second flask was similarly inoculated with C. albicans 67324. Both flasks were incubated for 48 hours as stationary cultures at 37 C. The spent broths were then filter sterilized (Millipore 0.45 mu pore size) and a two-fold serial dilution extending to 1:128 in 16x150 mm test tubes was prepared and inoculated with the alternate organism. After 72 hours the organisms in each tube were quantified by serial dilution in 0.85% saline. Blood agar pour plates were seeded with 0.1 ml of each dilution and incubated for 24 hours at 37 C. Results are presented as arithmetic means of duplicate counts and expressed as colony forming units per ml (CFU/ml).

In the second trial, Ps. aeruginosa 100 and C. albicans 67324 were inoculated into separate flasks, 1 ml of an overnight BHI culture was added to 200 ml of BHI and incubated at 37 C for 24 hours. The flasks were then cross

inoculated with the alternate organism using 1 ml of an overnight BHI culture. The flasks were sampled at 24, 48, 72 hours and 6 days. One-tenth ml of each was used as inoculum for duplicate blood agar plates. After 24 hours incubation at 37 C the plates were examined qualitatively for the presence of Pseudomonas and Candida.

The third trial consisted of three 500 ml Erlenmeyer flasks each containing 100 ml of BHI broth. The first flask was inoculated with 1 ml of Ps. aeruginosa 100 standardized to contain 25 cells per ml. The second flask was inoculated with C. albicans standardized to contain 24 cells per ml. The third flask received 1 ml each of the Pseudomonas and the Candida standardized inocula. The flasks were incubated at 37 C for 30 days and sampled at regular intervals. Ten-fold serial dilutions ranging from $1x10^{-3}$ to $1x10^{-8}$ were made and smeared onto MH agar and Molybdate Agar plates (Hospital Service Technology, North Andover, Mass.) for quantification (expressed as CFu/ml).

Overlay Technique

Detection of interference in liquid cultures proved to be an unwieldy technique. This difficulty was overcome by adopting the agar overlay method, the virtue of which lies in its simplicity and sensitivity. The first method employed was that of Osman's (26) which utilized a 6 ml nutrient base layer in a standard petri plate covered with 3 ml of

semi-solid (0.67%) agar previously seeded with two drops of an overnight broth culture of the test organism. The medium after solidification was placed in an incubator at 37 C for 90 minutes to dry. Drops of filtrate from effector strains of antagonistic microorganisms were placed on the surface and when dry, the plates were inverted and incubated at 37 C overnight. This technique lacked sensitivity to detect weakly antagonistic effector strains and was modified after the following studies were completed.

1. Base layer components. Three modifications were examined for enhancement of overlay zone development. The first compared nutritionally complete MH medium to Nutrient agar (NA, Difco). Ten ml of each medium were pipetted into standard petri plates and after hardening, 3 ml of 0.8% agar was used for the overlay. The second examined the effect of pH ranging from 4 to 8. Acid reactions of pH 4 and 6 were prepared by adding 2 N tartaric acid dropwise to sterile liquid (40 C) medium. Alkaline media with a pH reaction of 8 was prepared by the addition of 1 N sodium hydroxide. Ten ml of each adjusted preparation were pipetted into standard petri plates, allowed to harden, and the agar overlay applied as before. The third utilized five different base layer volumes of MH agar (5, 10, 15, 20 and 25 ml per plate). After hardening, a 3 ml agar overlay was applied.

In all of the above, the overlay consisted of 0.8% bacteriological grade agar prepared in 100 ml of distilled water seeded with 1 ml of an overnight broth culture of C. albicans 67324. When solidified, the overlay surface was spotted dropwise with sterile spent broth of Ps. aeruginosa 100. After overnight incubation at 37 C, zones of inhibition were measured with metric calipers.

2. Filtrate application and overlay agar volume. Using a base layer of MH agar (10 ml), both 3 and 6 ml volumes of overlay agar were compared for zone development. C. albicans 67324 was seeded as before and the agar allowed to solidify. Using a microliter syringe (Hamilton Model 710-LT, The Hamilton Co., Reno, Nevada), 10, 20, 30, 40 and 60 microliter volumes of filtrate were applied to the plates prepared with 3 ml of overlay agar. All plates were incubated overnight at 37 C and zones of inhibition measured.

3. Effector strain filtrate. Two hundred ml of BHI broth contained in a 500 ml Erlenmeyer flask was inoculated with 1.0 ml of an overnight BHI broth culture of Ps. aeruginosa 100 and incubated at 37 C for 14 days as a stationary culture. A very viscous gel accumulated during this time which required primary filtration with coarse Whatman No. 1 rapid flow filter paper prior to membrane filter sterilization (Millipore, 0.45 um pore

size). The resulting preparation, unless used immediately, was stored at -20 C.

To replenish the supply of filtrate, the preparation procedure was repeated at regular intervals throughout the experimentation. A pool was established and all filtrate harvested was added to it; the pool was labeled "Pool X." As needed, the pool was thawed at room temperature and an aliquot removed. The pool was then promptly refrozen and maintained at -20 C.

4. Modified overlay technique: final version. Ten ml of MH agar was poured into standard petri plates and allowed to solidify. Then 3 ml of 0.8% bacteriological grade agar seeded before solidification (40 C) with 1 drop of an overnight BHI broth culture of Candida albicans was used as the overlay. This layer was allowed to set and dry with the petri plate cover slightly ajar for 30 minutes at room temperature. Drops of Pseudomonas filtrate prepared as previously described on sterile blank paper discs (BBL, Baltimore, Md.) saturated with filtrate were applied to the surface. The plate covers were left slightly ajar until the filtrate had dried, then the covers were closed and the plates incubated at 37 C overnight. Zones of inhibition were measured in millimeters diameter.

Characterization of the Antagonistic Phenomenon

Using the overlay technique, further studies of the antagonistic phenomenon were implemented to characterize the Candida-Pseudomonas interaction and to ascertain the conditions which enhance the antagonism of Pseudomonas and the susceptibility of Candida. Also explored was the antagonistic effect of Pseudomonas on other microbial species.

1. Interaction: variation among strains. The Pseudomonas and Candida strains listed in Table 1 were screened to select those exhibiting maximum interference. The 11 strains of Pseudomonas and 12 strains of Candida were evaluated as effector and test strains, respectively. The degree of interference was judged on a scale of 0 to 3 to facilitate the selection process. The test and effector strains selected were used exclusively in the studies that followed.

2. Antagonistic spectrum toward other microbial species. Eleven fresh clinical isolates, randomly selected to serve as test organisms for Pseudomonas, were inoculated into sterile BHI broth (8 ml) and incubated overnight at 37 C. These test species included: Ps. iodium, S. viridans, S. pyogenes, S. Faecalis, S. aureus, E. coli, Herella vaginicola, Proteus mirabilis, K. pneumoniae and C. albicans. One drop of each broth culture was added to 3 ml of overlay agar in the procedure that followed.

3. Conditions favoring the production of active filtrate. This series of experiments examined the conditions which enhanced the antagonistic effect of Pseudomonas. Three 250 ml Erlenmeyer flasks containing 25, 50 and 150 ml of BHI broth were prepared and inoculated with 1 ml of an overnight BHI broth culture of Ps. aeruginosa 100. The flasks were incubated as stationary cultures at 37 C and 3 ml aliquots were harvested at the following times: 1, 2, 4, 6, 9, 12, 14 and 20 days. The aliquots were sterilized by membrane filtration and assayed using the overlay technique previously described. As in all of the procedures that follow, only discreet zones of complete inhibition were measured and recorded.

Incubation temperature variables were evaluated to establish the optimum for inhibitory activity. Three 500 ml Erlenmeyer flasks each containing 100 ml of BHI broth were inoculated with 1 ml of an overnight broth culture of Ps. aeruginosa 100 and incubated for 14 days at 30, 34, and 37 C. Aliquots were harvested, filtered and assayed using the overlay technique.

The formulation pH of BHI broth (7.4) was compared to adjusted levels of pH 6.4 and pH 5.4 to ascertain its effect on spent broth activity. Three 500 ml Erlenmeyer flasks each containing 200 ml of BHI broth were adjusted to the desired pH using 2 N tartaric acid. They were each

inoculated with 1 ml of an overnight BHI broth culture of Ps. aeruginosa 100 and incubated at 37 C for 14 days. After harvesting, the sterile filtrate was assayed for activity using the overlay technique.

Five media were evaluated for their potential as spent broth substrates: BHI (0.2% glucose), BHI (1.0% glucose), Pseudomonas F broth, Pseudomonas P broth and Nutrient broth (Difco); the latter three media contained no glucose. One hundred ml volumes of each were prepared in 500 ml Erlenmeyer flasks and inoculated with 1.0 ml of an overnight broth culture of Ps. aeruginosa 100 BHI. The flasks were incubated for 14 days at 37 C, harvested, filtered and assayed for activity.

4. Candida susceptibility as a function of incubation time. Two 500 ml Erlenmeyer flasks each containing 100 ml of BHI broth and inoculated with 1 ml of an overnight BHI culture of C. albicans 67324 were prepared. One flask was incubated for eight days and the other for one day. Both flasks were harvested, filtered and assayed simultaneously. The standard overlay procedure was followed.

Characterization and Purification of the Active Principle

1. Cell free extract. Ps. aeruginosa cells grown in 1300 ml of BHI broth for 5 days at 37 C were harvested by centrifugation at 8000 x g for 10 minutes in a Sorvall

RC-2B refrigerated centrifuge. The supernatant was discarded and the pellet of cells suspended in distilled water, 0.1 g (wet weight)/ml. The suspension was disrupted by 5 minutes of sonic oscillation (Biosonic, Bronwill Scientific Co.). The preparation was then passed through a French pressure cell (Wabash Metal Products, Wabash, Indiana) at 5000 lb/in^2 after which the cell debris was removed by centrifugation at 6500 x g. The supernatant was retained for assay of anti-candidal activity.

2. Dialysis. Spent broth was dialyzed using two types of membrane tubing: natural cellulose (Fisher Scientific, Pittsburgh, Pa.) which retains molecules with a molecular weight exceeding 12,000 and regenerated cellulose (Fisher Scientific) retaining molecular weights exceeding 3,500. Twenty ml of filtrate (Pool X) added to each dialysis sac was dialyzed for 24 hours in 500 ml of distilled water at 6 C. Aliquots were removed and spotted on overlay agar along with an untreated sample.

3. Trypsinization. One ml of freshly prepared 5% trypsin (Schwarz/Mann, lot #3910) was added to 9 ml filtrate (Pool X) and incubated at 37 C for 24 hours. Another 9 ml of filtrate with 1 ml of saline added, instead of trypsin, served as a control sample. The preparations were assayed using the standard overlay technique.

4. Thermal stability. The heat stability of the inhibitory compound produced by Pseudomonas was evaluated after heating aliquots of filtrate at 56, 68, and 100 C for 45 minutes and storing at 6 and -20 C for 12 days.

5. Stability to acid, light and desiccation. A 1.0 ml aliquot of filtrate (Pool X) was treated with 1N H_2SO_4 until pH 5 was obtained. After 15 minutes, 0.5 ml was removed and neutralized with 2 N NaOH to pH 8 and held for 15 minutes. An untreated aliquot of filtrate pH 8.3 (natural pH) served as a control sample. All preparations were then assayed for activity.

Stability to light and desiccation was determined by spotting cellulose polyacetate electrophoresis strips, 1" X 6" (Gelman Instrument Co., Ann Arbor, Michigan), with 30 microliters of filtrate. The strips were allowed to dry and remain exposed to the air and artificial light of the laboratory for five days. After exposure, the strips were appressed to the overlay agar surface along with control strips spotted with fresh filtrate. After incubation overnight at 37 C, the strips were removed and the plates re-incubated for 24 hrs for further growth of Candida.

6. Zone electrophoresis. Separation of the bio-active principle was attempted by electrophoresis on cellulose acetate strips (Sepraphore III) using an electro-

phoresis chamber (Gelman Instrument Co., Ann Arbor, Michigan). Twenty microliter samples of Pseudomonas filtrate (Pool X) were applied to the strips and allowed to dry. The strips were moistened in buffer and stretched across the chamber. A 0.25 microliter sample of RBY reference dye solution (Gelman Instrument Co.) was then applied to one end of a control strip and 175 volts were applied to the system for 45 minutes. The strips were removed and cut in half, each piece was then appressed to the surface of a freshly seeded C. albicans overlay agar plate and incubated for 24 hours at 37 C. The strips were then removed and the plates reincubated for an additional 24 hours to enhance the growth of Candida.

7. Extraction technique. To determine the polar nature of the active agents responsible for inhibition of Candida, three organic solvents were used. Nine 100 ml extraction flasks, each containing 20 ml of filtrate (Pool S) were divided into three groups. The first group was adjusted to pH 3, and the second to pH 7 using 1N H_2SO_4. The third group was adjusted to pH 9 using 2 N NaOH. The solvents used were hexane, diethyl ether and butanol (analytical grade). Twenty ml portions of each solvent were added to one flask of each group and after 2 minutes of hand shaking, the phases were allowed to separate. The aqueous phase was retained and extraction repeated, after

which the aqueous phase was separated, placed in a petri dish and allowed to air for 30 minutes. The nine preparations were then assayed for residual activity.

A second extraction scheme was followed using only chloroform as the solvent. Six ml of filtrate, adjusted to pH 2 using 6 N HC1 were extracted with 6 ml of $CHC1_3$. The $CHC1_3$ phase, after separation, was evaporated for 3 hours in a watch glass containing 4 blank paper discs. The aqueous phase remaining after extraction was adjusted to pH 10 using 6 N NaOH and extracted again with $CHC1_3$. The discs were then assayed for activity using the overlay technique.

The third approach to the selection of solvents was based on the observation of crystalline inclusions within colonies of Pseudomonas cultured on MH agar. After 4 to 6 days incubation, the agar plates were examined under low microscopic magnification (100 X), various organic solvents were added and dissolution of the crystals was noted.

The information gained from these preliminary studies prompted the investigation to follow an extraction scheme for the phenazines (Fig. 1) as reported by Chang and Blackwood (5). Ten microliters of extract were spotted on thin-layer chromatography plates coated with 0.25 mm of SIL G-25 silica gel without gypsum (Brinkman Instruments,

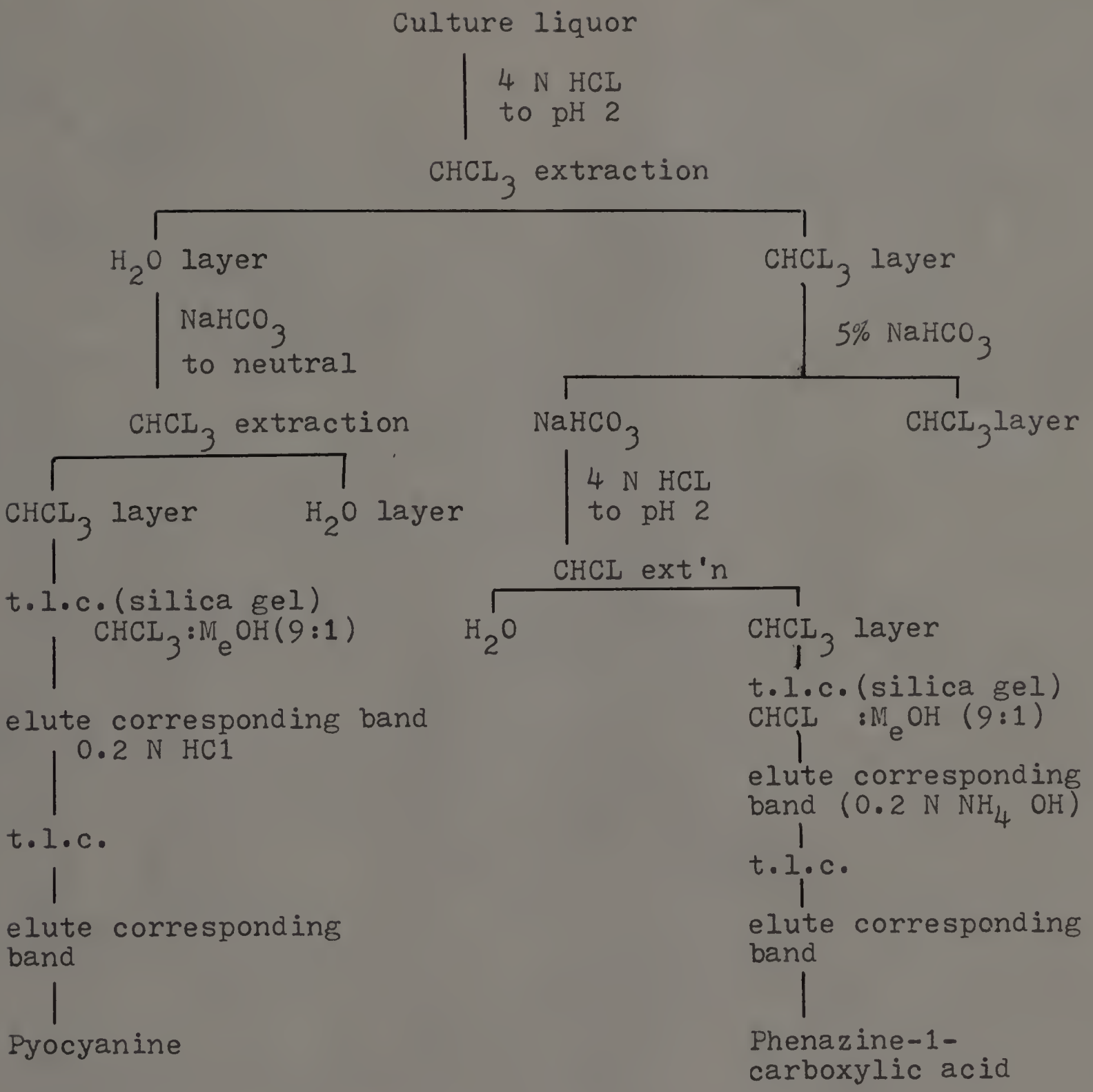


Fig. 1. Scheme for Separation of Two Phenazine Pigments.

Westbury, N.Y.). The bands were compared to reference pigments simultaneously chromatographed.

Identification Techniques

The band corresponding to Pyocyanine was re-chromatographed and eluted as previously described. This was followed by crystallization from cold $CHC1_3$ with the addition of petroleum ether. The melting point of the crystalline product was obtained with a microscope and a Kofler hot stage apparatus. The absorption maxima of the product in 0.2 N HC1 was measured on a Beckman DU model 2400 spectrophotometer. Both reference and isolated compounds were developed on Brinkman thin layer chromatograms using $CHC1_3$MeOH solvent mixture (9:1) and their rf values compared.

The band corresponding to phenazine-1-carboxylic acid was rechromatographed and eluted with 0.1 M NaOH. A melting point determination using a capillary tube apparatus was attempted, however, after reaching 360 C the product was presumed to be a salt. After redissolving in 6 N HC1 and extracting with $CHC1_3$ the preparation was allowed to evaporate. The pigment was then crystallized from cold MeOH and the melting point with a correction factor of 0.5 C was successfully determined. The crystalline appearance was compared to authentic samples of phenazine-1-carboxylic acid. The absorption maxima were measured in

0.2 N HC1. Following this the sample was chromatographed along with authentic samples using the same TLC procedure as described above for pyocyanine.

Biological activity of all bands was verified by scraping from the thin-layer chromatogram visualized with an ultraviolet lamp (Blak-Ray, model UVL-22, 366 nm wavelength, Ultraviolet Products, Inc., San Gabriel, Calif.). The scrapings were collected in a pasteur pipette packed with a small quantity of acid-washed glass wool to prevent loss of the powder. The band corresponding to pyocyanine was eluted with 0.1 N HC1 while that corresponding to phenazine-1-carboxylic acid was eluted with 0.1 N NaOH. Each eluate was absorbed on three blank paper discs and when dry were assayed for inhibitory activity using the standard overlay technique. Inhibition zones were compared to those obtained with authentic pyocyanine and phenazine-1-carboxylic acid. Both reference compounds were prepared in four concentrations (250, 25, 2.5 and 0.25 mcg/ml) in 0.1 ml of 0.1 N NaOH and added dropwise to the overlay agar surface.

Reference Compounds

Pyocyanine was purchased from Schwarz Mann, Orangeburg, N.Y. Phenazine-1-carboxylic acid was a gift from Frederick Helliman, Department of Organic Chemistry,

the University of Leeds, Leeds, Scotland. The organic solvents and other chemicals used were analytical grade.

R E S U L T S

Overlay Technique

Five variables were examined in the development of this technique and are summarized in Table 2. It can be seen that acidification of the base layer resulted in complete loss of inhibitory activity. On the other hand, MH medium at pH 7.4 induced a larger inhibitory zone than that obtained at pH 8.0.

The overlay agar and base layer volumes were inversely proportional to the resulting zone size. Some difficulty was experienced in preparing a 3 ml overlay of uniform depth. The problem was alleviated by pouring the overlay shortly after the base layer had solidified and was still warm. The volume of filtrate applied appeared to be stoichio-metrically related to the inhibition zone obtained. Results of the media evaluation indicated that the more enriched MH agar induced larger zones of inhibition than those obtained with nutrient agar.

Conditions for Interaction

As revealed in Table 3, serial dilution of inhibitory filtrate was accompanied by a progressively increased growth response by C. albicans. On the other hand, Ps. aeruginosa exhibited a diminished growth response in serial dilution of C. albicans filtrate. These findings indicate

TABLE 2

OVERLAY TECHNIQUE VARIABLES AND THEIR EFFECT ON INHIBITION ZONE DEVELOPMENT

Variables evaluated	Inhibition zone[a]
pH (base layer)	
4.0	0
6.0	0
7.4	15.7
8.0	13.6
Overlay volume (ml)	
3.0	9.1
6.0	6.0
Base layer volume (ml)	
5.0	20.8
10.0	17.0
15.0	14.5
20.0	11.0
25.0	8.5
Filtrate volume (ul)	
10	0
20	6.0
30	8.5
40	12.0
60	14.5
Medium	
MH	15.7
Nutrient agar	13.1

[a]Zone diameter in millimeters.

TABLE 3

EFFECT OF FILTRATE DILUTION ON
GROWTH OF ASSOCIATE ORGANISM

Reciprocal of filtrate dilution	Plate counts	
	Candida (CFU 10^5)	Pseudomonas (CFU 10^6)
2	6.0	146.5
4	14.0	80.5
8	36.5	75.0
16	32.0	53.5
32	35.0	62.5
64	46.5	57.5
128	63.0	55.5

that in associative cultures, Candida is inhibited while Pseudomonas is stimulated.

The intent of this study was to determine whether the inhibitory activity of the filtrate was due to the development of physiological and chemical changes (including substrate depletion) in spent broth or the elaboration of an antibiotic substance. Since the serial dilution of the spent broth was accompanied by a commensurate growth response of Candida, it was hypothesized that an antibiotic substance was responsible for the inhibition.

The second experiment examined the effect of inoculating each species into established cultures of the alternate species (Table 4). While Ps. aeruginosa was capable of establishing itself in an actively growing culture of C. albicans, Candida could not be established in an actively growing culture of Ps. aeruginosa.

To further confirm this antagonistic interaction, growth curves of the two organisms growing separately and together were plotted. In this case the mixed culture was established by simultaneous inoculation, a method outlined by Guze, et al (7). As can be seen in Fig. 2, C. albicans survived but exhibited a diminished population when compared to growth in pure culture. The foregoing studies suggest that Candida, if given time to establish

TABLE 4

ISOLATION OF C. ALBICANS AND PS. AERUGINOSA FROM ASSOCIATIVE CULTURES ORIGINALLY ESTABLISHED WITH A SINGLE SPECIES

Time after assoc. culture inoculated	Culture originated as:	Organism recovered	
		C. albicans	Ps. aerug.
1 day	Candida	+[a]	+
	Pseudomonas	-	+
2 days	Candida	+	+
	Pseudomonas	-	+
3 days	Candida	+	+
	Pseudomonas	-	+
6 days	Candida	+	+
	Pseudomonas	-	+

[a] + growth
- no growth

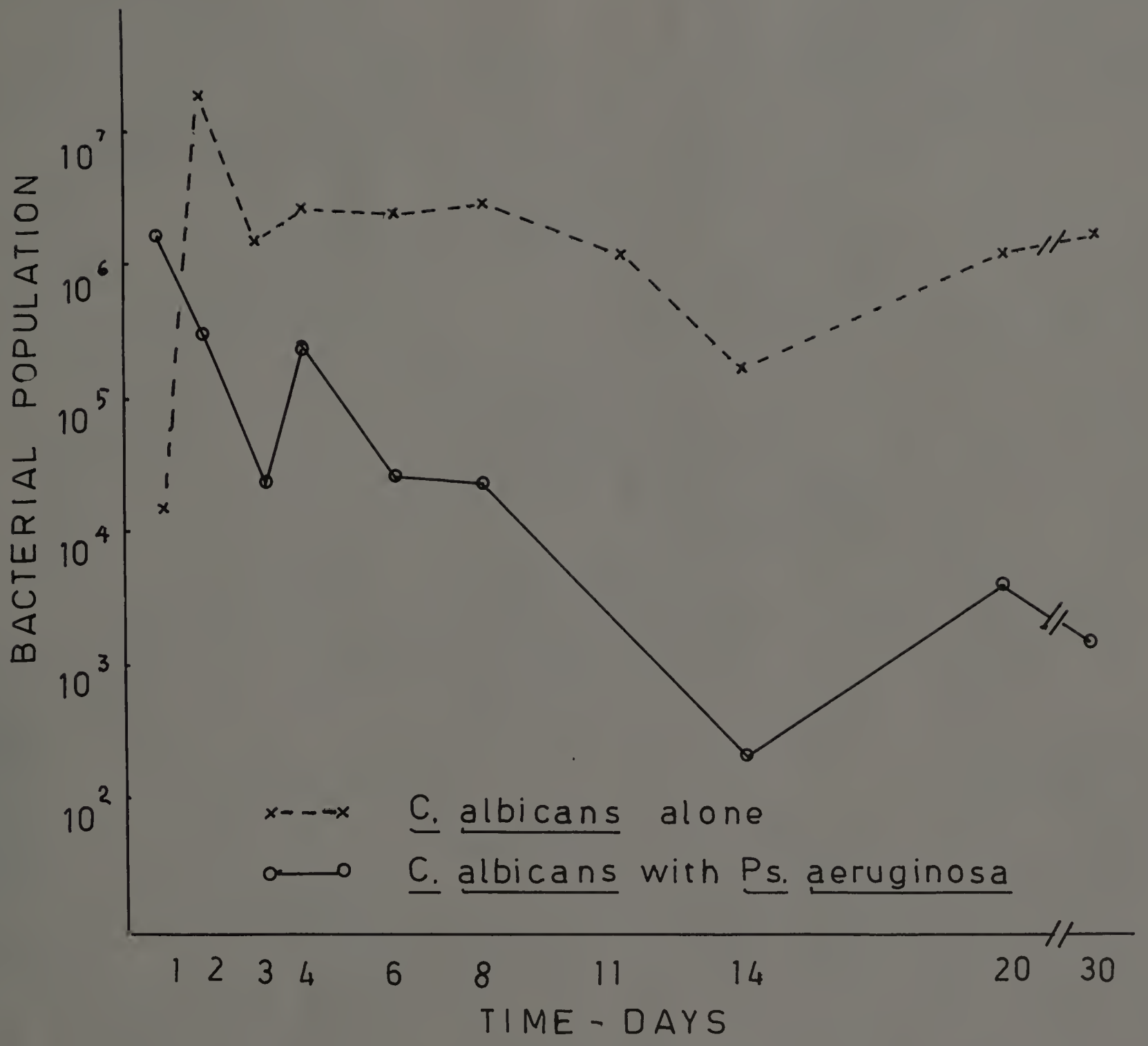


Figure 2. Growth of C. albicans alone and in the presence of Ps. aeruginosa ,

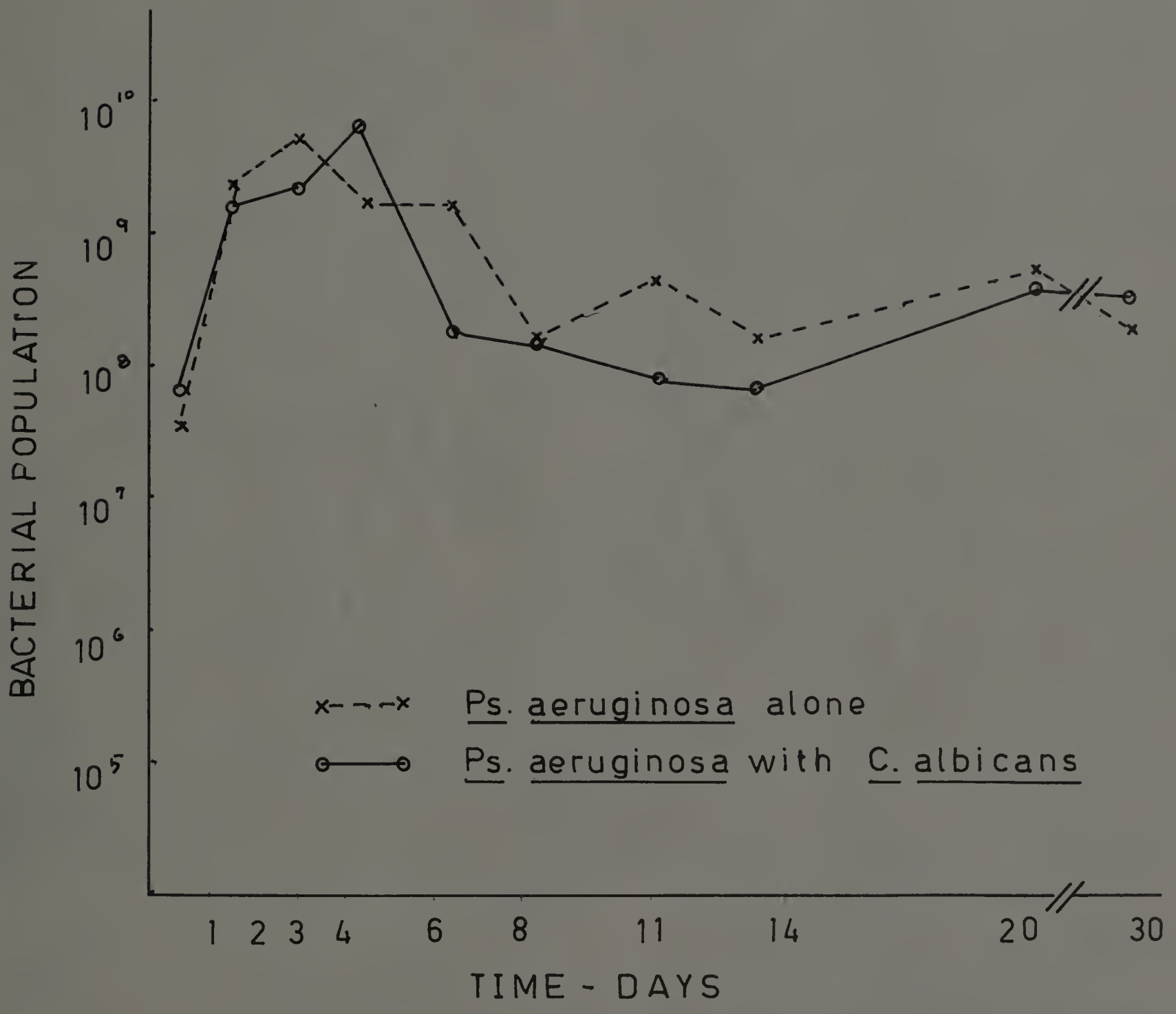


Figure 3 Growth of Ps. aeruginosa alone and in the presence of C. albicans.

logarithmic growth can adapt to the introduction and development of Pseudomonas.

The antagonistic spectrum of Pseudomonas toward randomly selected organisms is shown in Table 5. The diversity of susceptible organisms further supports the hypothesis that the active principle was an antibiotic and not a bacteriocin or a phage.

The survey of conditions optimum for the yield of the hypothesized antibiotic indicated that the incubation time for stationary cultures must extend beyond 4 days (Table 6). The highest yield was obtained from a 14 day old culture although activity was still detectable at 20 days. These findings are similar to those of other workers who reported that old cultures of Pseudomonas were highly "bacteriocidal" to many organisms (39, 41). Also shown in Table 6 is the correlation between yield and medium volume. Since no activity was observed in the very shallow (25 ml) culture flask, it appears that the redox potential may influence the synthetic pathway of the antibiotics.

The data in Table 7 show that of the 3 temperatures evaluated for the incubation of Pseudomonas, 30 C yielded the most potent filtrate. Approximately 84% of this activity was detected at 34 C and only 50% at 37 C. These observations are in accord with those made by Chang and Blackwood while investigating the phenazine pigment (5).

TABLE 5

ANTIMICROBIAL SPECTRUM OF
PSEUDOMONAS FILTRATE

Test Organism	Range	Mean[2]
Candida albicans	14-15[b]	14.4
Escherichia coli	14-16	15.0
Herellea vaginicola	12-13	12.6
Klebsiella pneumoniae	0	0
Proteus mirabilis	16-17	16.8
Pseudomonas iodinium	0	0
Staphylococcus aureus	15-17	16.0
Streptococcus faecalis	0	0
Streptococcus pyogenes	0	0
Streptococcus viridans	0	0

[a]Zone diameter of five replicate plate counts.

[b]Zone diameter in millimeters.

TABLE 6

THE EFFECT OF INCUBATION TIME AND MEDIUM VOLUME ON THE DEVELOPMENT OF FILTRATE POTENCY

Incubation Time (days)	Medium Volume		
	25 ml	50 ml	150 ml
1	0	0	0
2	0	0	0
4	0	indiscreet zones	
6	0	7.8[a]	7.0
9	0	7.7	9.1
12	0	5.6	7.4
14	0	8.6	10.9
20	0	6.2	8.4

[a]Zone diameter in millimeters.

TABLE 7

THE EFFECT OF INCUBATION TEMPERATURE ON FILATRATE POTENCY DEVELOPMENT

Pseudomonas Incubation Temperature C	Range	Mean
30	13-16[a]	15.2
34	10-14	12.8
37	5-10	7.2

[a]Zone diameter in millimeters.

Kluyver (19) made a similar observation in his studies using Ps. aureofaciens as the effector strain. He noted that 30 C was optimal for both growth and pigment (antibiotic) production, while 35 C yielded satisfactory growth but no detectable pigment.

No difference in activity was noted in the filtrate prepared from BHI broth adjusted to pH levels of 5.4, 6.4 and 7.4. On the other hand, evaluation of various media and the resultant filtrate activity revealed that only BHI broth yielded detectable inhibition. While it has been reported that glucose or glycerol in concentrations of 1-8% are necessary for quantity production of the phenazine pigments (9), it should be noted that none of the other media contained carbohydrate.

The final condition examined was the effect of incubation time on the susceptibility of C. albicans to pseudomonas pigments. Table 8 shows three growth phases of Candida (exponential, stationary and death) which were used to seed the agar overlay. While there was essentially no difference in the data obtained between the stationary and death phases, the susceptibility in the exponential phase was approximately 10% less than that of the stationary phase. This observation substantiates the data obtained from associative cultures showing the adaptability of

TABLE 8

THE RELATIONSHIP BETWEEN INCUBATION TIME OF C. ALBICANS AND ITS SUSCEPTIBILITY TO PSEUDOMONAS FILTRATE

C. albicans Growth Phase	Susceptibility	
	Range	Mean
Exponential 24 hrs.	14-17[a]	15.5
Stationary 48 hrs.	17-18	17.3
Death 28 days	17-19	17.6

[a]Zone diameter in millimeters.

Candida in its early growth phases.

Agents Responsible

While the foregoing results of interactive phenomena seem to indicate that an antibiotic agent was responsible, more conclusive evidence was established upon the completion of the extraction and purification techniques. Characterization prior to these techniques showed that the active principle in the filtrate was not an enzymatic or proteinaceous agent. Trypsinization did not affect the bioactivity nor did any of the thermal treatments. The substance was stable on exposure to light and dessication and the molecular weight as indicated by dialysis was less than 3,500. Definitive data were not obtained from studies using cell-free extract nor was the separation technique using zone electrophoresis successful. Neither investigation was pursued further and the data are not presented.

The activity was completely lost on acidification of the filtrate to pH 5. After reversal to 8.3 (original pH), activity was restored and comparable to the untreated control sample. These findings supported the observations made in the development of the overlay technique where an acid pH base layer completely neutralized antibiotic activity (Table 9).

Crystalline inclusions within colonies of *Pseudomonas* growing on MH agar proved to be soluble in $CHCl_3$, sparingly

soluble in butanol and not soluble in hexane as observed by microscopic examination (100 X).

The extraction scheme of Chang and Blackwood yielded two bio active products: (1) deep blue rhembic crystals soluble in $CHCl_3$ and hot water, consistent with pyocyanine and (2) yellow, needle-like crystals, soluble in $CHCl_3$ and alkali, consistent with phenazine-1-carboxylic acid. Although a number of other spots were observed when these crude extracts were chromatographed only these two compounds were active against C. albicans. The physiochemical properties obtained after the final crystallization are compared with authemtic samples (tables 10, 11). As can be seen, both the isolated and the reference compounds behaved identically. As further proof, derivatives were prepared. A semi-carbazone from pyocyanine and an amide from phenazine-1-carboxylic acid showed rf values similar to derivations of the isolated pigments.

TABLE 9

THE EFFECT OF ACIDIFICATION ON THE DEVELOPMENT OF FILTRATE POTENCY

pH manipulation	Inhibition zone	
	Range	Mean
8.3 5.0	no inhibition	no inhibition
8.3 5.0 8.3	16 - 20[a]	18.6
8.3 (control	18 - 21	19.6

[a]Zone diameter in millimeters.

TABLE 10

PROPERTIES OF EXTRACTED AND AUTHENTIC PYOCYANINE PIGMENT

	Isolated pigment	Authentic pyocyanine
Appearance	dark blue needles	dark blue needles
Melting point C with correction factor 0.5 C	132-132.5	132-132.5
Absorption maxima mu in 0.2 N HCL	390, 278	390, 279
Solubility	$CHCl_3$, hot water	$CHCl_3$, hot water
TLC: rf	.55	.55
TLC: rf deriv. semi-carbazone	.65	.63

TABLE 11

PROPERTIES OF EXTRACTED AND AUTHENTIC
PHENAZINE-1-CARBOXYLIC ACID

	Isolated pigment	Authentic pigment
Appearance	yellow needles	yellow needles
Melting point C with correction factor 0.5 C	239	239
Absorption maxima mu in 0.2 N HCL	368, 253	368, 251
Solubility	$CHCl_3$, NaOH	$CHCl_3$, NaOH
TLC: rf	.68	.66
TLC: rf deriv carboxamide	.61	.61

D I S C U S S I O N

This investigation commenced with the observation in the clinical laboratory that colonization of the respiratory tract by both Pseudomonas aeruginosa and Candida albicans rarely occurred. Coexistence, when observed, was for a brief interval. Ultimately however, one species would be pre-empted by the other; moreover, the succession of species was observed to progress with predictability from Candida to Pseudomonas.

During the past two decades, these organisms have come to be recognized as opportunists and are being isolated with increasing frequency from the immunologically incompetent or "compromised" host. The unfavorable prognosis given patients suffering from infections caused by these two species arises from the lack of effective antimicrobial agents and not from their inherent virulence. Treatment, in many cases, is based on resolution of an underlying disease or condition which generally accompanies these infections. The possibility of manipulating antagonistic interaction between these two organisms and its benefit was an exciting prospect and prompted the present investigation.

The appearance of antagonism on culture plates of clinical material is a common laboratory observation.

Clinical specimens rarely yield growth of a single organism; pathogens are often accompanied by indigenous "contaminants" and culture plates incubated for 72 hours reveal a microbial flora distinctly different from that seen after 24 hours incubation. Overgrowth of slower growing species, the predominance of one species over another and the effect of antibiotic therapy all contribute to the phenomenon of apparent antagonism. However, when the preliminary observation of antagonism between Pseudomonas and Candida was made these factors were discounted. The colonial characteristics of Candida and Pseudomonas are strikingly different and in mixed cultures their presence would be evident. Both organisms grew readily on simple media, producing distinct colonies after 24 hours incubation. Since Candida is relatively resistant to antibiotics, its suppression by these agents in clinical specimens would be unlikely.

From these preliminary observations it was proposed that Pseudomonas was antagonistic towards Candida. The associative culture studies indicated that the inhibition of Candida was due to an antibiotic agent and not to nutrient depletion or restrictive physiological conditions. The "flip-flop" technique further confirmed this contention. Since there was no direct contact between Candida and Pseudomonas, the possibility that inhibition was due to

phage particles was discounted.

The next observation was the appearance of crystalline inclusions within colonies of Pseudomonas using low microscopic magnification. Various organic solvents were added dropwise to these colonies to ascertain the solubility of the crystals. Chloroform was found to be a very effective solvent for these crystals.

The experiments conducted during the filtrate preparation stage of this study revealed that several antibiotic pigments were produced during extended cultivation of Ps. aeruginosa. At least two of these pigments were found to be inhibitory to C. albicans and belonged to the phenazine series of compounds.

It was determined however, that the yield of these pigments could be more readily obtained from liquid cultures. Stationary flasks incubated at 37 C for 2 to 4 days showed development of a deep blue-green pigment in the upper layers of the broth. When the incubation was continued for 14 days, the bottom layer became greenish-yellow until finally a yellow-brown color developed throught the medium. At the same time, a very evident intractable slime developed throught the medium which disintegrated to some extent during the extraction process. Nevertheless, it must be removed by coarse filtration prior to membrane sterlization.

The bioactive phenazine pigments obtained by extraction were identified as phenazine-1-carboxylic acid and pyocyanine. These pigments were readily separated in the extraction process by manipulating the pH of the spent medium (generally 8.0 to 8.5 after 14 days). After acidification of the aqueous phase to pH 2.0, phenazine-1-carboxylic acid formed a precipitate which was soluble in $CHCl_3$. Pyocyanine, on the other hand, formed an insoluble precipitate when the medium was made alkaline (pH 9) and could be extracted with $CHCl_3$.

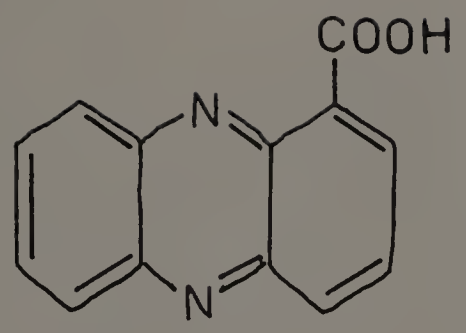
Chromatographic separation at this point revealed both extracts to be heterogeneous. The extract containing pyocyanine, developed on TLC plates, was accompanied by six other bands as detected by ultraviolet light. That containing phenazine-1-carboxylic acid was accompanied by seven other bands. Bromcresol-blue spray, used to detect carboxylic acids, was applied to developed plates and a positive reaction indicated the position corresponding to phenazine-1-carboxylic acid. The organic nature of these compounds was confirmed by charring with concentrated sulphuric acid and heat. Of all the bands produced, these two were among the most prominent and when eluted were the only ones exhibiting detectable antagonism towards Candida. Though bioactivity was not detectable in the remaining bands, it is possible that several other phenazines were

present. Their concentration was perhaps too low to be detectable by the assay method used.

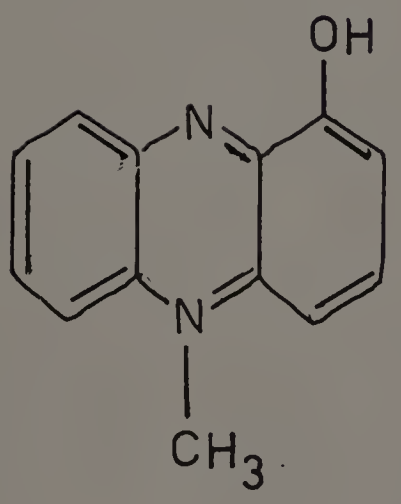
Presently, about 30 phenazines have been isolated from natural sources. A literature search disclosed that although pseudomonads are the most prominant source known; Actinomycetes, algae and other diverse species synthesize these compounds (1). It has further been shown that antibiotic effects are common with the phenazines and the mechanism of action appears due to intercalation with deoxyribonucleic acid (13).

The biosynthetic pathway of the phenazines has been elucidated by Hellstein (12). When radioactive labeled shikimic acid was supplied to Ps. aeruginosa, 9% was incorporated into phenazine-1-carboxylic acid and 7% into pyocyanine. The structural formulas for these two compounds is shown in Fig.4 .

The biological activity of these two phenazines was found to be pH dependent. Under acid conditions, phenazine-1-carboxylic acid was apparently insoluble and biologically inactive whereas the sodium salt of this acid possessed marked activity, the inhibition zones produced by the salt were distinctly clear with a discreet periphery and could be accurately measured. Furthermore, elution of this compound from silica gel of the TLC plates was readily accomplished with methyl alcohol containing 1% NH_4OH or



PHENAZINE 1 CARBOXYLIC ACID



PYOCYANINE

Figure 4. Structural formulas of phenazine-1-carboxylic acid and pyocyanine.

0.1 N NaOH while methyl alcohol or $CHCl_3$ alone were not nearly as effective.

Pyocyanine is considered a neutral compound and its bioactivity as a function of pH was not as evident as that seen with phenazine-1-carboxylic acid. Methyl alcohol and $CHCl_3$ were both good solvents. Inhibition of Candida as observed on overlay agar plates was less evident than that seen with phenazine-1-carboxylic acid. Within the inhibition zone Candida's presence was obvious. The inhibition zones of both compounds were examined under low magnification (100 X) and Candida cells were readily observed: the cell population was greater in the case of pyocyaine and budding was more obvious. When these zones were subcultured on fresh Sabourauds Dextrose agar, growth with original vitality was regained. This suggests that in the case of Candida these compounds are fungi-static. Moreover, with weak or dilute preparations, inhibition was difficult to detect or was entirely overlooked.

When filter paper discs were impregnated with these compounds and the discs placed in close proximity on the overlay agar surface, approximately two centimeters apart, a reinforcing effect was observed (Fig. 4). The asymmetric inhibition pattern had the appearance of synergism, although this was not investigated further. The area around pyocyanine was virtually without affect except where influenced by phenazine-1-carboxylic acid.

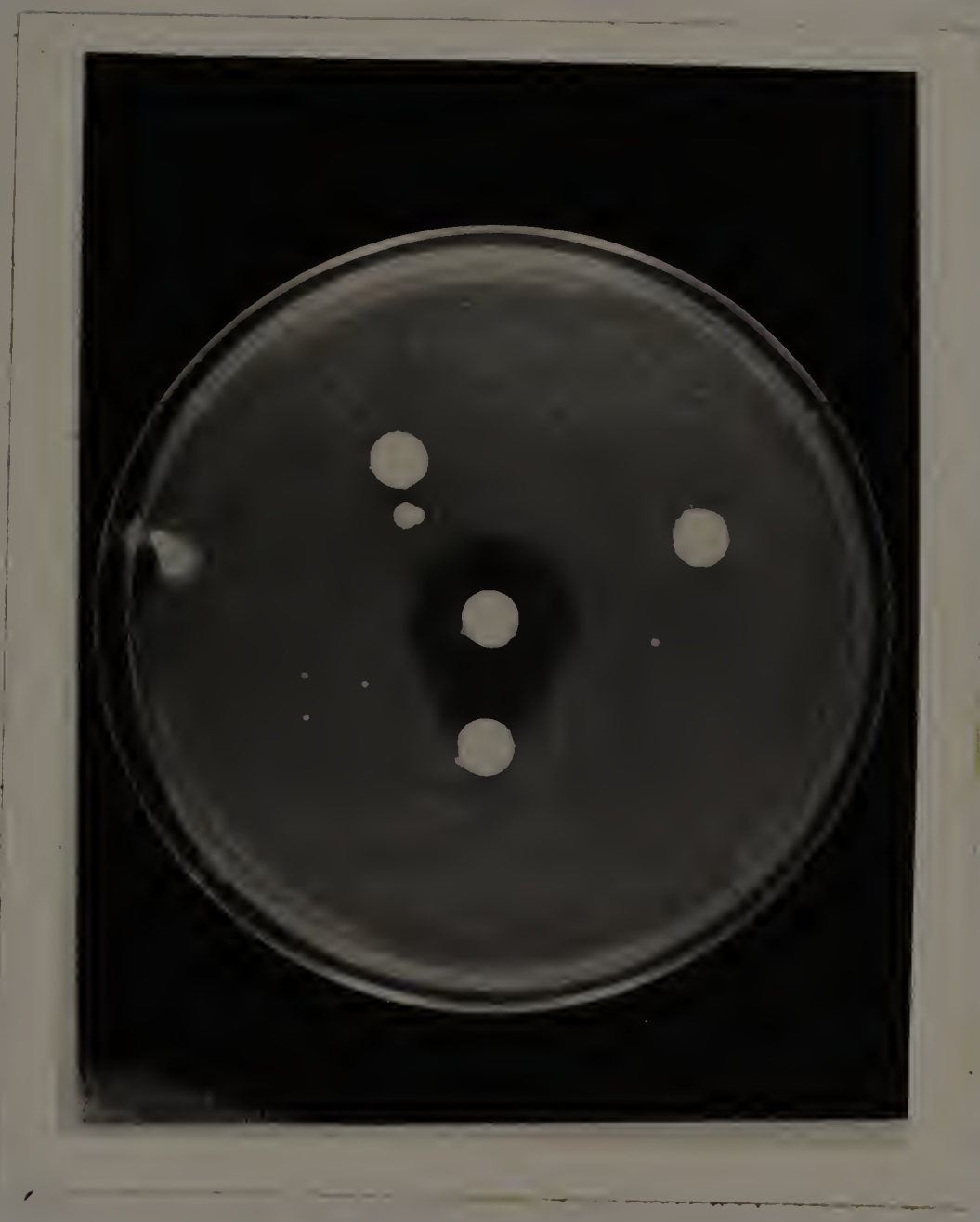


Fig. 5. Apparent synergism between phenazine-1-carboxylic acid and pyocyanine. Note the asymmetric zone of inhibition around the central disc containg phenazine-1-carboxylic acid caused by the nearest satellite disc containing pyocyanine.

Another consideration was the biological activity of these phenazines *in-vivo* and its relationship to pH. Since antagonism appears to be dependent on an alkaline environment, it is conceivable that interaction might not take place in the oral cavity or the vagina when these habitats are excessively acidic (28). The *in-vitro* data at pH 6.0 and below showed complete loss of anti-candidal activty (Table 2). The concentration of serum glucose and its conversion by the indigenous flora to acid end-products may be the major determinant of an individual's tendency to develop an infection by either one or both of these organisms. While *Candida* infections are commonly associated with high concentrations of serum glucose, low concentrations of this substrate accompanied by a more neutral pH may favor the succession of *Pseudomonas*.

However, the measurable advantage to the patient of a *Pseudomonas* rather than a *Candida* infection is highly questionable. Since both organisms are the cause of severe disease, it is inconceivable that modern medicine would risk the purposeful introduction of one pathogen to suppress another.

This report should reaffirm our respect for opportunistic infections. Evidence has been presented here that suggests that these infections can occur in

succession, Candida followed by Pseudomonas, each of which is a test of the patient's immunological competence and medicine's therapeutic capability.

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